PATENT APPLICATION
Docket No. 356830.00300
Former Docket No. 18810-80364
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Examiner: J. A. Goldberg
JULIA LJUBIMOVA et al.	Art Unit: 1634
Serial No.: 09/741,550	
Filed: December 19, 2000	
For: USING OVEREXPRESSION OF LAMININ ALPHA 4 SUBUNIT AS A DIAGNOSTIC AND PROGNOSTIC INDICATOR OF MALIGNANT TUMORS	

SECOND DECLARATION OF JULIA LJUBIMOVA UNDER RULE 132 (37 CFR 1.132)

I, JULIA LJUBIMOVA, declare as follows:

1. I am a named inventor on the present application and a citizen of the United States residing at 11419 Dona Pegita Dr., Studio City, California 91604

2. I obtained my M.D. degree from Kiev Medical University, Kiev, Ukraine in 1983 and a Ph.D. degree in Oncology-Pathology from the Kavetsky Institute for Oncology Problems, Academy of Sciences of Ukraine, Kiev, Ukraine. My thesis was entitled "Molecular peculiarities of DNA in malignant melanomas and benign nevi of human skin".

3. I have held positions of Staff Research Associate in the Department of Reproductive Medicine at the University of California San Diego and Research Scientist in the Department of Surgery and Neurosurgical Institute at Cedars-Sinai Medical Center, Lost Angeles, California. I have also held appointments as an Adjunct Assistant Professor in the Department of Surgery at the University of California Los Angeles.

4. My current title is Director, Molecular Oncology Maxine Dunitz Neurosurgical Institute, Cedars-Sinai Medical Center. I also serve as a Clinical Assistant Professor in the Department of Neurosurgery at the University of California Irvine.

5. I have over twenty published scientific publications and have given numerous lectures and scientific presentations on cancer and related subjects.

6. I have a great deal of experience with malignancies both at an experimental and clinical level. During the studies that led to the above-named patent application I realized that α4 laminin undoubtedly had significance in many, if not all, malignancies partially because of its relationship to vasculature in malignancies. Complete clinical studies are difficult and time consuming. However, prior to filing the above named application I was able to perform preliminary tests on malignant tumors not of brain origin. Although human breast cancer appears quite different from brain cancer, I discovered that like brain malignancies, malignant tumors of the breast overexpress α4 laminin.

7. Since the filing of the present patent application I have expanded my observations on malignant breast tumors into a full study that parallels the instant patent application. As I show in the data presented below, the results for malignant breast tumors are very similar to those for malignant brain tumors. However, the picture with breast tissue is somewhat more complex because of the presence of

several different tissue types including ductal cells within a breast biopsy whereas a brain biopsy lacks the same diversity of tissues. As explained and demonstrated in paragraphs 12 and 13, below, there is some α4 laminin expression in normal ductal tissue but not in normal breast vasculature. There is significant α4 laminin expression in vessels of breast cancers—this corresponds to the situation in brain cancer. Because breast tissue and brain tissue are inherently dissimilar tissues, the similarity in α4 laminin expression supports the theory that such expression is a marker of malignancy and increases the invasiveness of the malignancy—apparently through some effect on the vasculature.

8. To demonstrate the present invention with breast malignancies A total of 45 human breast tissue samples were used for Western blot analysis and immunohistochemistry, including 14 normal breast tissues, 4 ductal carcinoma *in situ* (DCIS), 23 primary invasive ductal carcinoma of breast and 3 brain metastases of breast cancer.

9. For Western Blot analysis twenty-eight of the tissue samples were used: 10 normal breast tissues, 4 DCIS, 11 primary invasive ductal carcinomas and the 3 brain metastasis of breast cancer. Tissue samples were snap-frozen in liquid nitrogen by a pathologist immediately after surgery. Proteins were separated using 10% Tris-Grysin SDS-PAGE (Invitrogen, Carlsbad, CA) under reducing conditions. Lysates of human glioma T98G, known to express Laminin-8 (Ln-8) were used as a positive control. The gels were blotted onto nitrocellulose membrane (Invitrogen, Carlsbad, CA). The membranes were probed with monoclonal antibodies followed by chemiluminescent detection using the Immun-Star™ AP kit with alkaline phosphatase-conjugated secondary antibodies (BIO-RAD, Hercules, CA). Antibodies were used to the Ln α4 chain (mAb 8B12)[15], β1 chain (rAb LT3)(upstate, Lake Placid, NY) and β2 (clone C4)(LAB VISION, Fremont, CA). Antibody to β-actin (SIGMA, St. Louis, MO) was used to control for equal loading of

gel lanes. The intensities of the bands of interest were expressed relative to the β-actin band intensities for the same specimens, using the AlphaImager™ 2000 densitometer (Alphainnotech, Inc., San Leandro, CA). Background was subtracted from each reading before determining the intensity ratios.

10. We compared the expression of α4, β1 and β2 chains of laminin using Western blot analysis. The expression of the α4 chain of laminin that is one of the constituents of Ln-8 is various in tumor tissues and also in corresponding normal tissues (Fig. 1). However, the expressions of β2 chains are higher in normal tissues than in cancerous tissues. Furthermore, the intensity patterns of β2 expressions in normal tissues are similar to those of α4 expression. On the other hand, the expression of β2 is lower or absent in the tissues of primary tumor and metastasis. These results tend to show that the occasional strong expression of α4 in normal tissues correspond to Laminin-9 (Ln-9) but not Ln-8. It is believed that variability is due to the mix of tissue types included in a given biopsy. In contrast, the expression level of β1 chains was higher in tumor tissues and the expression levels are ranked in tumor grade dependent manner. Though the expression of β1 chains of laminin is present in normal breast tissues, the level of expression is lower than in cancerous tissues. To take the expression of α4 and β2 into consideration, the expression pattern of the combination with α4 and β1 is more predominant in cancerous tissues than normal tissue. Specifically in brain metastasis of breast cancer, the expression of the combination with α4 and β1 is higher than in those of primary breast or normal breast tissues. However, from the viewpoint of histology, multiple types of cell components are included in the breast tissues. Therefore, to clarify the localization of the each component of laminin chains and to identify the laminin complex, we used immunohistochemistry.

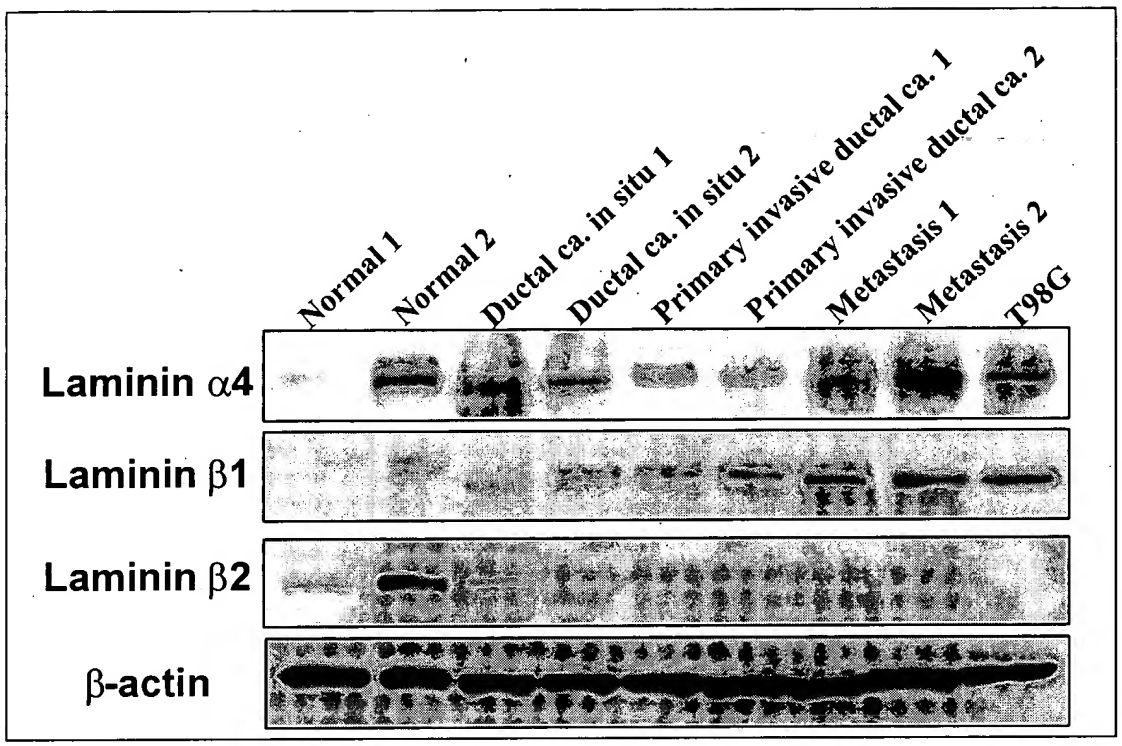

_*Figure 1.* Western blot analysis of normal breast tissues, ductal carcinomas in situ, invasive ductal carcinoma tissues and brain metastases from the primary breast cancer for Laminin α4, β1 and β2 chains (typical results of 8 / 28 samples are shown). The expression of Laminin α4 chain, a constituent of Laminin-8, varies in tumor tissues and also in corresponding normal tissues. However, the expression of β2 chain, a constituent of Laminin-9, is relatively high in normal tissues, but is very low or non-existent in breast cancer tissues. The levels of β2 expressions in each normal tissue are similar to those of α4 expressions. These results may show that any strong expression of α4 in normal tissues corresponds to Laminin-9 but not to Laminin-8. In contrast, the expression of Laminin β1 chain, another constituent of Laminin-8, is high in brain metastases and primary invasive ductal carcinoma, but the expression decreases in ductal carcinomas in situ and is very low in normal tissues. Laminin α4 chain migrates at 200 kDa; Laminin β1 chain, at 230 kDa; Laminin β2 chain, at 190 kDa; β-actin, at 47kDa. T98 glioblastoma cell line that only expresses chains of Laminin-8 (α4 and β1) is used as a positive control.

11. Thirty-eight tissue samples were used for immunocytochemical analysis: 14 normal breast tissues, 4 DCIS, 15 primary invasive ductal carcinoma and 3 breast cancer metastases in the brain. Tissue samples were snap-frozen in liquid nitrogen by a pathologist immediately after surgery and embedded in OCT

compound, and 8-mm sections were cut on a cryostat. Indirect immunofluorescence, photography, and routine negative controls were obtained. Polyclonal and monoclonal antibodies were used against laminin chains α4 (clone 1F9), β1 (clone LT3), and β2 (clone C4). Rabbit polyclonal antibodies against laminin α4 chains (LG1–3) were also used. The secondary cross-species absorbed fluorescein- and rhodamine-conjugated donkey anti-mouse, anti-rat, and anti-rabbit antibodies were from Chemicon International (Temecula, CA). The polyclonal antibodies against human von Willebrand factor (Sigma, St. Louis, MO) were used for endothelial cell detection. Monoclonal antibodies against cytokeratin 8/18 (biomeda, Foster, CA) were used for epithelial cell detection. Monoclonal antibodies were used as straight hybridoma supernatants or at 10-20 mg/ml when purified, and polyclonal antibodies were used at 20-30 mg/ml.

12. The expression of each of the laminin chains was investigated using 36 clinical samples (normal 14, in situ 4, invasive breast cancer 15, brain metastasis 3) to determine α4 and β1 co-localization. The expressions of α4 chains in invasive ductal carcinoma and brain metastatic tissues were always co-localized with β1 chains. While, the expressions of α4 chains were co-localized mainly with β2 chains in normal breast tissues. These data indicate that Ln-8 (α4β1γ1) expresses in breast tumor tissue but Ln-9 (α4β2γ1) expresses in normal tissue. As shown in Table 1, Ln-8 expressed 93.3% primary invasive breast cancer tissues and 100% brain metastasis. The Ln-8 expressing tissues were decreased in DCIS and we detect low signal of Ln-8 in normal tissue (Table 1). In contrary, the expressions of Ln-9 were detected in all normal tissues including 92.8% of vessels BMs (basement membranes), 100% of mammary duct's BMs (Table 1) and adipose tissues (data not shown). The reason why the expression of Ln-9 were variable in normal tissues seems to be because of the large variety of histological components in normal tissues including mammary ducts, vessels, connective tissues and adipose tissues. The BMs of mammary ducts that are involving Ln-9 partially disappeared in DCIS

and are absent in invasive ductal carcinoma and metastatic tissues. But only 6.7% in primary invasive breast tumor tissue and none of brain metastasis expressed Ln-9 without Ln-8 at the BMs of vessels (Table 1).

Table 1.

Histological diagnosis	Number of cases *n*	Laminin-8 *n* (%)	Laminin-8/-9 *n* (%)	Laminin-9 *n* (%)
Normal breast tissue	14	1 (7.1%)	1 (7.1%)	12 (85.7%)
Ductal carcinoma in situ	4	1 (25%)	2 (50%)	1 (25%)
Invasive ductal carcinoma	15	11 (73.3%)	3 (20%)	1 (6.7%)
Metastases	3	3 (100%)	0	0

13. To identify the localization of Ln-8 in the breast cancer tissues, we co-stained $\alpha 4$ or $\beta 1$ chains with endothelial marker (von Willebrand factor) or epithelial marker (Cytokeratine 8/18). The $\alpha 4$ or $\beta 1$ chains were co-localized with the von Willbrand factor (VWF) in invasive ductal carcinoma and metastatic tissues but were not co-localized with cytokeratine. However, the $\beta 2$ co-localized with basement membranes of mammary ducts and the vessels in normal tissues. In addition, though we also detect low expression of $\beta 1$ in normal vessels of some tissue samples, the expression was very low in comparison with the expression of that in cancerous tissues. In DCIS, the expressions of both Ln-8 and Ln-9 were seen at the basement membranes of microvessels independently. These data suggest that the switch from Ln-9 to Ln-8 in the process of breast cancer progression starts at early phase of carcinogenesis. Moreover, the results clearly shows Ln-8 is dominant in the tumor microvessel basement membrane in breast cancer tissues, while Ln-9 is predominant the normal breast vessel basement membranes.

I hereby declare under penalty of perjury that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed this 28th day of July 2004 at Los Angeles, California .

J. Ljubimova

Julia Ljubimova

07/28/2004

I hereby declare under penalty of perjury that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed this 28th day of July 2004 at Los Angeles, California .

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Julia Ljubimova


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LOCUS       HS142L7                 190778 bp    DNA     linear   PRI 04-MAR-2003
DEFINITION  Human DNA sequence from clone RP1-142L7 on chromosome 6q21,
            complete sequence.
ACCESSION   Z99289
VERSION     Z99289.1  GI:3168992
KEYWORDS    HTG.
SOURCE      Homo sapiens (human)
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            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 190778)
  AUTHORS   Tubby,B.
  TITLE     Direct Submission
  JOURNAL   Submitted (04-MAR-2003) Wellcome Trust Sanger Institute, Hinxton,
            Cambridgeshire, CB10 1SA, UK. E-mail enquiries:
            humquery@sanger.ac.uk Clone requests: clonerequest@sanger.ac.uk
COMMENT     On May 30, 1998 this sequence version replaced gi:2578058.
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            Center: Wellcome Trust Sanger Institute
            Center code: SC
            Web site: http://www.sanger.ac.uk
            Contact: humquery@sanger.ac.uk
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            During sequence assembly data is compared from overlapping clones.
            Where differences are found these are annotated as variations
            together with a note of the overlapping clone name. Note that the
            variation annotation may not be found in the sequence submission
            corresponding to the overlapping clone, as we submit sequences with
            only a small overlap as described above.
            This sequence was finished as follows unless otherwise noted: all
            regions were either double-stranded or sequenced with an alternate
            chemistry or covered by high quality data (i.e., phred quality >=
            30); an attempt was made to resolve all sequencing problems, such
            as compressions and repeats; all regions were covered by at least
            one plasmid subclone or more than one M13 subclone; and the
            assembly was confirmed by restriction digest, except on the rare
            occasion of the clone being a YAC.
            The following abbreviations are used to associate primary accession
            numbers given in the feature table with their source databases:
            Em:, EMBL; Sw:, SWISSPROT; Tr:, TREMBL; Wp:, WORMPEP; Information
            on the WORMPEP database can be found at
            http://www.sanger.ac.uk/Projects/C_elegans/wormpep This sequence
            was generated from part of bacterial clone contigs of human
            chromosome 6, constructed by the Sanger Centre Chromosome 6 Mapping
            Group.  Further information can be found at
            http://www.sanger.ac.uk/HGP/Chr6
            RP1-142L7 is from the library RPCI-1 constructed by the group of
            Pieter de Jong. For further details see
            http://www.chori.org/bacpac/home.htm
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